

## ORIGINAL PAPER

# EXPRESSION OF $\beta$ -CATENIN AND ITS CORRELATION WITH METASTATIC PROGRESSION OF ESOPHAGOGASTRIC JUNCTION ADENOCARCINOMA

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The evaluation of  $\beta$ -catenin expression in adenocarcinoma of the esophagogastric junction and its influence on cancer progression.

Sixty-one patients who were diagnosed with adenocarcinoma of the esophagogastric junction were examined. We evaluated  $\beta$ -catenin distribution in the cell membrane and the cell nucleus in adenocarcinoma of the esophagogastric junction type 1 and type 3.

Our findings showed lack of a statistically significant difference in evaluation of adenocarcinoma type 1 and type 3 aggressiveness. However, we found a statistically significant association with the T and N stage, although we did not find an effect of them on patient survival.

Patients with cellular membrane and cell nucleus staining comprise a group of patients with higher risk of malignancy progression in adenocarcinoma of the esophagogastric junction types 1 and 3.

**Key words:**  $\beta$ -catenin, esophago-gastric junction, adenocarcinoma.

## Introduction

In the last decades the incidence of esophagogastric junction adenocarcinoma has been rising. At the same time, the incidence of squamous cell carcinoma has been declining or remains the same. Despite radical surgical and radio/chemotherapy, survival rates are not satisfactory and the risk and evaluation of carcinoma relapse are uncertain [1, 2]. The identification of patients with higher risk of carcinoma relapse would facilitate therapeutic treatment strategy significantly.

Due to progress in immunohistochemistry and molecular diagnostics, the role of cell adhesion molecules has become more accurate and understandable.  $\beta$ -catenin was described as an element of the E-cadherin/catenin complex. In healthy cells catenin is united with adherin, but its action depends on APC (adeno-

matous polyposis coli) and phosphorylation by serine-threonine kinase GSK-3B. In the event of mutations, the phosphorylation process becomes disturbed and  $\beta$ -catenin passes into the cell's nucleus with LEF (lymphocyte enhancer factor)/TCF (T cell factor). It turns out that such action may decode the reaction which through MMP-7 (matrix metalloproteinase 7), cyclin D1, MDR 7 (multidrug resistance gene) and later through AD-1 and fra-1 may start the process of carcinogenesis or tumor progression [3, 4]. The role of the E-cadherin/catenin complex in adenocarcinoma of the esophagogastric junction is unclear. The aim of this study is to assess the influence of  $\beta$ -catenin expression on the metastatic potential of adenocarcinomas localized in the esophagogastric junction.

## Material and methods

### Patients

Sixty-one patients with confirmed adenocarcinoma of the gastroesophageal junction were included in the study. According to the Siewert and Stein classification [5] they were divided into three groups: patients with adenocarcinoma related to Barrett esophagus (AEG type I), adenocarcinoma of cardia (AEG type II) and subcardial adenocarcinoma (AEG type III). Only patients with type I and type III adenocarcinoma were included in this study because sometimes differentiation between type 2 and 3 in stage T3 or T4 makes difficulties in assessment [5]. The TNM UICC and Lauren classification was used for assessment of the disease stage [6, 7]. Patients with AEG1 tumors underwent transmediastinal esophagectomy, proximal stomach and lymphadenectomy in the posterior mediastinum and celiac axis. An extended total gastrectomy with resection of the distal esophagus and D2 lymphadenectomy with transhiatal lymphadenectomy of the inferior mediastinum was the procedure for patients with AEG3 tumors. In total 1130 lymph nodes were removed during surgery, on average 22 lymph nodes per patient (range 8-58 lymph nodes). In 60 cases surgery was radical (R0), while 1 case was classified as R1. The mean follow-up time for the 61 patients was 27.4 months, with a range of 1-96.7 months. Table I shows clinical and histopathologic outcomes of patients.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue specimens were cut into 6  $\mu$ m thick section which were placed onto silanized slides, then deparaffinized and rehydrated. Antigen retrieval was performed by means of a citric acid solution, pH 6.0, which was heated in a microwave oven for 1  $\times$  15 min, at 650 W. Endogenous peroxidase activity was blocked with a 0.2% hydrogen peroxide solution. The primary antibody was the  $\beta$ -catenin specific HEC-D1 monoclonal antibody (Takara Biomedicals, Takara Shuzo Japan) that was incubated for 1.5 h at room temperature at a dilution of 1 : 1000. Detection of the bound antibody was carried out with the avidin-biotin complex peroxidase method (ABC Elite kit, Eliot Burlingame CA), followed by staining with the peroxidase substrate 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma GmbH, Deisenhofen Germany). A light hematoxylin stain was used as the counterstain. As a negative control, the primary antibody was replaced with phosphate-buffered saline. Normal esophagus mucosa with membranous (M+) and no nuclear (N-) expression in the tissue section served as a positive control. Membranous (M) and nuclear expression of  $\beta$ -catenin was assessed. Membranous

staining for  $\beta$ -catenin was classified as a normal (3+), reduced (moderate) (2+), weak (1+) or negative (0). The percentage proportion of stained cells with complete membranous staining was recorded in the following categories: 0 – 0-10%, 1 – >10-40%, 2 – 40-90%, 3 – > 90%. In addition, the presence and percentage of tumor cells exhibiting clear nuclear staining for  $\beta$ -catenin were noted and classified as 3+ (> 67% of the tumor cells), 2+ (> 33% and < 67% of the tumor cells), 1+ (< 33% of the tumor cell nuclei).

### Statistics

The correlations between the immunohistochemical expression of membranous  $\beta$ -catenin, nuclear catenin staining, survival, grade of differentiation, lymph node metastases, tumor size, and Lauren classification were statistically compared using the  $\chi^2$  test or Fisher's two-tailed test. Survival probabilities were calculated by the Kaplan-Meier method and compared by the log rank test. P values < 0.05 were considered to be statistically significant.

## Results

Sixty-one patients diagnosed with adenocarcinoma of the esophagogastric junction; 32 of them with type 1 and 29 with type 3 (Table I) were analyzed. Among patients with type 1, in 21 we found reduction of cell membrane staining and/or cell nucleus staining with anti- $\beta$ -catenin antibody. In 11 of them staining was normal and in 2 of them the reaction was negative. In patients with type 3 findings were as follows: in 21 patients we found cell membrane staining reduction and/or staining of the cell nucleus. In 8 patients staining was normal and in 1 patient the reaction was negative. Staining of cell nuclei was found in 9 patients (in 5 of them there was normal cell membrane staining) with type 1 and 11 (in 3 of them there was normal cell membrane staining) in type 3 (Table II). Among patients with staining reduction in type 1 (AEG 1) carcinomas, in 16 of them we found an intestinal type, in 2 diffuse type, and in 3 a mixed type (according to Lauren's classification). In patients with type 3 (AEG 3) in 15 patients we found intestinal type, in 4 patients a diffuse type and in 1 patient a mixed type. Staining of cell nuclei was found in 6 patients with an intestinal type, in 2 patients with a mixed type and in 1 patient with a diffuse type. Among patients with type 3 (AEG 3), in 8 of them we found an intestinal type, in 2 patients a diffuse type and in 1 patient a mixed type. In 12 patients we found both staining of the cell membrane and a nuclear reaction (M+, N+). In 22 patients we found a reduction in cell membrane staining (M+, N+) and in 8 patients cell nucleus staining. In 19 patients staining of the cell membrane was classified

**Table I.** Clinical and pathomorphological data of patients with AEG1 and AEG3

GROUP	AEG1	AEG3	TOTAL
N	32	29	61
Sex M : F	26 : 6	23 : 6	61
Age	45-75	33-78	33-78
Follow-up	27.7 (1-96.7)	26.6 (1-91.7)	27.4 (1-96.7)
Differentiation Grade G1 : G2 : G3	1 : 16 : 15	0 : 16 : 13	1 : 32 : 28
Lauren type ( I : M : D)	26 : 3 : 2	19 : 4 : 6	45 : 7 : 9
TNM	T1N0M0 11 T2N0M0 3 T3N0M0 2 T1N1M0 1 T2N1M0 6 T3N1M0 7 T2N2M0 2	T1N0M0 5 T2N0M0 6 T3N0M0 1 T1N1M0 1 T2N1M0 8 T3N1M0 1 T4N1M0 1 T2N2M0 1 T3N2M0 5	T1N0M0 16 T2N0M0 9 T3N0M0 3 T1N1M0 2 T2N1M0 14 T3N1M0 8 T4N1M0 1 T2N2M0 3 T3N2M0 5
	32	29	61

AEG 1 – adenocarcinoma of gastroesophageal junction type 1; AEG 3 – adenocarcinoma of gastroesophageal junction type 3; Lauren (I : M : D) – Lauren (intestinal : mixed : diffuse)

as stage 3, and in 41 patients no nucleus staining was found.

In 3 patients the results were negative. Staining reduction correlated with lymph node metastases ( $p = 0.048$ ) and with T stage ( $p = 0.041$ ). However, staining distribution had no effect on patients survival. Its connection with Lauren's classification and degree of carcinoma differentiation (Table II) was not established.

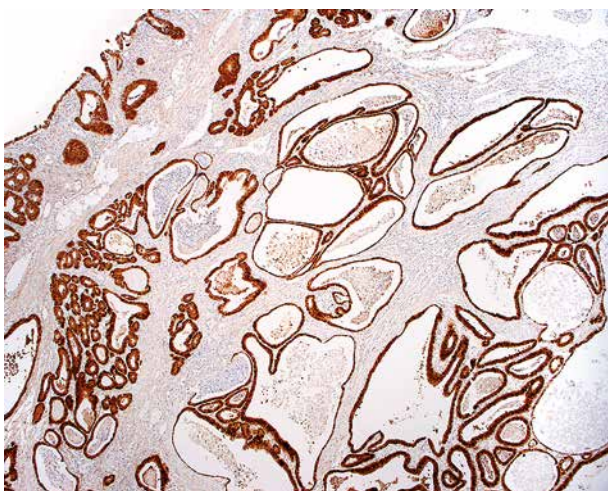
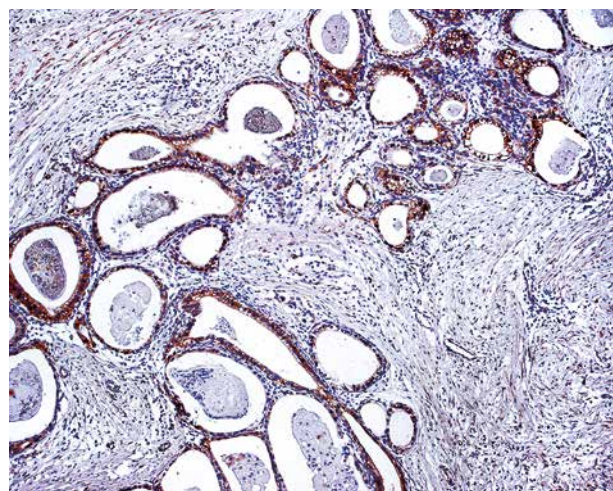
## Discussion

$\beta$ -catenin is a member of the cell adhesion molecules, where together with E-cadherin it forms a group of interepithelial glycoproteins responsible for carrying out the function of adhesion with other membrane proteins. It has various subcellular locations occurring in the cell membrane, cytoplasm, and cell nucleus. The E-cadherin/catenin complex not only plays a role in the cell adhesion process but also plays a key role in a signal transmission from the external environment and through reaction with receptors it may create a particular malignant phenotype. Presumably, it may play an important role in initiation as well as progression of malignancy, as evidenced by changes in staining of the cell membrane as well as the cell nucleus [8, 9, 10, 11]. Loss of the epithelium of a mucous membrane's integrity may be an hypothesis about the impact of this complex on phenotype change and malignancy progression.

It is considered that maintenance of cell adhesion is possible due to E-cadherin function connected with  $\beta$ -catenin in the cytoplasm. Krisdanatu and partners reported staining reduction of  $\beta$ -catenin in relation to differentiation and shorter survival, not finding it to be connected with T stage [9]. Wijahaven *et al.* reported that staining reduction was associated with T stage only. They did not observe its association with grade. There was no association between esophageal adenocarcinoma and carcinoma of a proximal part of the stomach [12]. In an earlier study, the authors analyzing 24 patients with adenocarcinoma of the esophagogastric junction observed a staining reduction in cell membrane, accumulation in cytoplasm (54%, 75%, 67%, 63%) and nucleus relocation (25%) for E-cadherin,  $\alpha$ -,  $\beta$ -,  $\gamma$ -catenin but without a correlation in relation to TNM classification [13]. In other reports, the authors note a strong association with shorter patient survival, in whom  $\beta$ -catenin staining reduction was found. Heaving realized that this feature constituted an independent prognostic factor, they stated that it may help in identification of patients with clinically negative lymph nodes, who are at risk of malignant disease progression. Some authors note that staining change or lack of it in patients with lymph node metastases may have a different character than in the original tumor, therefore they suggest that staining change may be the result of downregulation of other regulation [12, 14]. Bian *et al.* found that staining reduction is associated with

**Table II.** Correlation between  $\beta$ -catenin, nuclear catenin and prognostic factors in adenocarcinoma of the esophagogastric junction

	M(+) N(+)	M(+) N(-) (FIG. 1)	M(-) N(+) (FIG. 2)	P FOR T AND N
Aeg 1	6	12	3	
Lauren	4i, 1d, 1m	10i, 1d, 1m	2i, 1m	
G	G1-	G1	G1	
	G2 1	G3 5	G2 1	
	G3 5	G3 6	G3 2	
T	T1	T1 4	T1 1	
	T2 3	T2 3	T2 2	
	T3 3	T3 5	T3	
	T4	T4	T4	
N	N0 1	N0 3	N0 2	0.041
	N1 4	N1 9	N1 1	0.048
	N2 1	N2	N2	
Aeg 3	6	10	5	
Lauren	4i, 1d, 1m	7i, 3d	4i, 1d	
G	G1	G1	G1	
	G2 2	G2 6	G2 4	
	G3 4	G3 4	G3 1	
T	T1 1	T1	T1 2	
	T2 3	T2 8	T2 3	
	T3 2	T3 2	T3	
	T4	T4	T4	
N	N0 3	N0 1	N0 2	
	N1 2	N1 6	N1 3	
	N2 1	N2 3	N2	
Total	12	22	8	

**Fig. 1.** Immunohistochemistry in the surgical specimen – reduction of  $\beta$ -catenin in cell membrane staining – M(+) N(-). Objective magnification 20 $\times$ **Fig. 2.** Immunohistochemistry in the surgical specimen – staining of cell's nucleus M(-) N(+). Objective magnification 20 $\times$

the depth of infiltration, but did not find any connection with tumor differentiation or lymph node metastases, whereas the authors observed a significant correlation with longer survival [11]. In our opinion the decisive factor for survival was the T stage. The role of catenin remains unclear. Its location and accumulation in the cell are connected with Wnt-pathway activation (coded group of family genes). The Wnt gene family influences transmission of many signals in physiological processes such as cell differentiation, migration and transcription, but also its role in pathology is suggested, particularly in the process of carcinogenesis. The signals transmitted through Wnt are submitted by  $\beta$ -catenin and its action is regulated by the adenomatous polyposis coli APC/GSK-3B complex. So stabilized catenin, controlled by phosphorylation process GSK-3B remains in the cytosol, whereas in the case of stimulation, it is transported into the cell nucleus in the form of T-cell factor complex causing cyclin D1, c-Myc, PPAR-sigma, and MMP 7 stimulation. Myc causes the induction of telomerase transcription, affecting an unidentified factor releasing p27 and blocking the cyclin *cdk2*. It is considered that catenin degradation may prevent APC and e-adherin mutations. Wnt and catenin activation may be connected to the process of oncogenesis and malignancy progression as well [4, 15]. In this study,  $\beta$ -catenin staining distribution in esophageal adenocarcinoma (on the ground of Barrett's) type 1 and subcardial stomach carcinoma type 3, depending on severity, differentiation, and Lauren's classification, was compared. Reduction of staining intensity was observed in the membranous part, while normal staining was observed in the cell nucleus. In this study a statistically significant difference in staining reduction, metastatic potential, and differentiation degree dependent on T stage was found. Gunther *et al.* found that nuclear expression of  $\beta$ -catenin may have a different effect on the course of rectal and colon carcinomas [16]. Our studies confirmed incorrect staining of  $\beta$ -catenin in 39 (61) patients diagnosed with adenocarcinoma of the esophagus type 1 and type 3. The difference in staining for the individual types was not statistically significant. No statistically significant relation of staining reduction to patient survival or Lauren's classification was observed. Czyżewska *et al.* found a statistically significant dependence of  $\gamma$ -catenin staining, Lauren's classification intestinal type and degree of stomach cancer differentiation but did not find a correlation with survival. They found a strong correlation between stage of stomach cancer and metastatic potential [17]. Analyzing nuclear staining in tumor type 1 (AEG 1) and type 3 (AEG 3) and referring them to Lauren's classification, they found them in 13 (28%) patients in Lauren's type 1

(7 in AEG 1) and 6 in AEG 3. Lee *et al.*, analyzing patients with early stage stomach cancer, found that relocation, that is an increased level of  $\beta$ -catenin in the cytoplasm and cell nucleus, among patients with intestinal mucin phenotype in comparison with gastric mucin phenotype in stomach cancer, concerned 30% of patients. At the same time they found a lack of association between loss of APC heterozygosity and relocation, while attributing the role of hypermethylation in the process of  $\beta$ -catenin relocation [18]. Woo *et al.* reported that staining of the nucleus and relocation took place in diffuse type stomach cancer in 27% of patients. The above is not confirmed by Ogasawa *et al.* They reported that staining of the cell nucleus occurred more often in intestinal type stomach cancer than in diffuse type [19, 20]. It seems that  $\beta$ -catenin relocation into the cell nucleus is possible due to c-Myc and cyclin D1 activation [21]. Bondi *et al.*, analyzing colon cancer, did not find a correlation between  $\beta$ -catenin relocation and c-Myc or cyclin D1 activation. The authors noted that  $\beta$ -catenin relocation constituted metastatic potential while not noting this correlation in relation to c-Myc, cyclin D1 and  $\gamma$ -catenin. They assumed that the reason for that is metalloprotease, which is a target gene for the  $\beta$ -catenin/TCF pathway in colon cancer and can be activated independently from c-Myc and cyclin D1. Overexpression of MMP-7 is found in 80% of colon cancer cases and is an important factor in invasion and metastases [22]. The role of  $\beta$ -catenin relocation into the cell nucleus is emphasized by other authors, who prove its influence on malignant transformation in colon and pancreatic carcinomas [23, 24]. Incorrect  $\beta$ -catenin staining is observed in other organs such as liver, lungs, pancreas, and colon, although the mechanism of its activation can vary [25, 26, 27, 28]. Xianhua *et al.*, analyzing the survival in 262 patients diagnosed with non-small cell lung cancer observed incorrect  $\beta$ -catenin staining in 189 (76%) patients, in correlation with Wnt1, overexpression c-Myc, cyclin D1, and p53. In the analyzed group, patients in whom positive staining was found had significantly shorter survival, but in multifactorial analysis Wnt1 expression constituted an independent prognostic factor of survival [25, 29].

In conclusion, we observed a correlation of  $\beta$ -catenin reduction with T and N stage, without an influence on survival. Its relocation into the cell nucleus concerned 20 patients. However, the role of  $\beta$ -catenin in relation to progression of malignancy remains unclear. The biological effect of staining reduction provides for an increased metastatic potential.

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*The authors declare no conflict of interest.*

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